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Ubiquitination of Retinoblastoma Family Protein 1 Potentiates Gene-specific Repression Function*[S]

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Background: *Drosophila* Rbf1-mediated transcriptional repression of E2F1-dependent genes is canonically regulated by cyclin/cdk-mediated phosphorylation.
Results: Rbf1 ubiquitination and turnover is mediated by an evolutionarily conserved C-terminal degron that paradoxically enhances repression of E2F1-dependent genes.
Conclusion: Ubiquitination enhances Rbf1 repression potency in a promoter-specific manner.
Significance: Rbf1 ubiquitination presents a mechanism for regulatory discrimination of distinct physiological pathways.

The retinoblastoma (RB) tumor suppressor family functions as a regulatory node governing cell cycle progression, differentiation, and apoptosis. Post-translational modifications play a critical role in modulating RB activity, but additional levels of control, including protein turnover, are also essential for proper function. The *Drosophila* RB homolog Rbf1 is subjected to developmentally cued proteolysis mediated by an instability element (IE) present in the C terminus of this protein. Paradoxically, instability mediated by the IE is also linked to Rbf1 repression potency, suggesting that proteolytic machinery may also be directly involved in transcriptional repression. We show that the Rbf1 IE is an autonomous degron that stimulates both Rbf1 ubiquitination and repression potency. Importantly, Rbf1 IE function is promoter-specific, contributing to repression of cell cycle responsive genes but not to repression of cell signaling genes. The multifunctional IE domain thus provides Rbf1 flexibility for discrimination between target genes embedded in divergent cellular processes.

The RB[4] tumor suppressor protein functions as a crucial regulator of the G_1/S transition during cell cycle progression and thus plays a central role in restricting cellular proliferation (1). Consistent with this property, the RB1 gene is inactivated in a broad range of human cancers, often as a seminal event contributing to both cancer initiation and cancer progression (2). RB has been further implicated in the governance of diverse physiological processes, including differentiation and apoptosis, and as a central hub connecting these processes, RB activity is subjected to strict control by post-translational modification during normal growth and development (3, 4). Indeed, in many tumor types, upstream regulatory pathways governing RB are inactivated with similar frequencies as inactivation of RB itself, attesting to the importance of close supervision over RB function (5).

In an intricate network of gene control, RB and its related family members, p107 and p130, function as transcriptional repressors of diverse gene sets through interactions with members of the E2F family of transcriptional activator proteins (6, 7). RB family members govern apparently mutually exclusive physiological processes, notably cell cycle progression and apoptosis, and thus distinct regulatory mechanisms must ensure that RB-mediated induction of apoptosis does not ensue, even as RB proteins are periodically activated on cell cycle genes during normal proliferation (8). Canonical regulation of RB activity is governed by cyclin/cdk regulatory kinases (9–12). Timely phosphorylation blocks RB/E2F association and unleashes waves of E2F-mediated transcription that contribute to cell cycle progression (13). However, RB continues to reside at a number of genomic sites after cyclin/cdk-mediated deactivation (14, 15), revealing that cyclin/cdk activity does not universally derepress all RB target genes. Indeed, RB phosphorylation by p38 MAPK at a site that is not a target for cyclin/cdks can modulate RB-mediated repression of apoptotic response genes (8, 16). This model suggests that RB is subjected to a protein modification code that enables gene-specific outcomes, namely cyclin/cdk kinases regulate cell cycle-responsive promoters and stress-responsive kinases regulate apoptosis-responsive promoters.

In *Drosophila*, RB family proteins Rbf1 and Rbf2 interact with E2F transcription factors as corepressors, similar to their mammalian counterparts. *Drosophila* Rbf proteins are also controlled by a canonical phosphorylation mechanism through cyclin-cdk complexes (17, 18). Mutant rbf1 embryos show constitutive expression of PCNA and RNR2, two E2F1-regulated genes for DNA replication, and ectopic S-phase entry, indicating the importance of Rbf1 for arresting cells in G_1 phase (19). Rbf1 associates at numerous canonical E2F cell cycle-regulated genes in the early embryo (20, 21), indicating that key compo-

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[4] The abbreviations used are: RB, retinoblastoma; IE, instability element; PCNA, proliferating cell nuclear antigen; InR, insulin receptor; cdk, cyclin-dependent kinase; Ub, ubiquitin; NLS, nuclear localization sequence; DMSO, dimethyl sulfoxide; luc, luciferase.

nents of the RB regulatory pathway are evolutionarily conserved. However, in the embryo, Rbf1 also associates with numerous E2F1-independent target genes beyond the canonical cadre of E2F1-dependent target genes (22, 23). Many of these candidate E2F1-independent target genes encode components of signaling pathways, exemplified by the insulin receptor (InR), and whose expression is regulated independently of the cell cycle. Thus, *Drosophila* Rbf regulatory influence during development appears to extend beyond cell cycle progression and apoptosis to include cellular signaling, although in a mechanism likely independent of E2F1.

In addition to regulation by phosphorylation, Rbf proteins are subject to developmental regulation of their proteolytic turnover. Developmental regulation occurs in imaginal disc tissue (20) with stability controlled by the COP9 signalosome (24), a developmentally regulated complex that controls proteasome-mediated protein degradation via modulation of E3 ubiquitin ligase activity (25, 26). The COP9 signalosome is physically associated with Rbf1 and Rbf2, and depletion of COP9 subunits stimulates Rbf1 turnover (24). Rbf1 stability is influenced by a C-terminal instability element (IE) that positively contributes to both repressor destruction and repressor potency (20). The conservation of the IE in mammalian RB family proteins suggests that these pathways operate in higher eukaryotes; however, the function of the IE in integrating protein turnover and transcriptional control is poorly understood. Here, we show that the Rbf1 IE is sufficient to facilitate ubiquitination and turnover and directly mediates transcriptional repression. Strikingly, Rbf1 ubiquitination enhances E2F1-dependent PCNA repression but not E2F1-independent repression of InR transcription. Thus, the IE is a key protein motif directing promoter-specific activity of Rbf1. These studies reveal a novel level of regulatory discrimination within the RB protein modification code that enables gene-specific repression during development.

EXPERIMENTAL PROCEDURES

Expression Constructs—Generation of Rbf1 WT and mutant expression constructs was described previously (20). To generate GFP fusion proteins, enhanced green fluorescent protein (eGFP) cDNA was PCR-amplified from phs-eGFP and cloned into KpnI site of pAX vector. Two FLAG epitope tags were inserted 5′ of the stop codon. The C terminus and the IE of Rbf1 were made by site-directed mutagenesis. To minimize the differences among mRNAs transcribed from GFP fusion protein constructs, the first two amino acids of the IE were mutated into stop codons to generate GFP alone constructs. Tet fusion protein expression constructs were generated as described previously (27). Rbf1 WT and mutants were digested from pAX-rbf1 vector and ligated into KpnI and XbaI sites of pAX-Tet vector. The C terminus and the IE were amplified with KpnI and XbaI on the ends and inserted into pAX-Tet vector. To generate ubiquitin fusion proteins, the ubiquitin coding sequence was amplified using oligonucleotides with KpnI sites on both ends, and the amplicon was inserted into the KpnI site of the pAX vector. The C-terminal glycine residues at the junction were initially mutated to alanine to prevent ubiquitin removal by isopeptidases (Ub-Rbf1-ΔIE, see Fig. 6*D*) and then to isoleucine (Ub-Rbf1, see Fig. 6, *B* and C) to provide a more complete block to cleavage.

Luciferase Reporter Assay—Drosophila S2 cells were transfected using Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. Typically, 1.5 million cells were transfected with 100 ng of Ac5C2T50-luciferase reporter, 0.25 μg of pRL-CMV *Renilla* luciferase reporter (Promega), and 20 ng of one of pAX-Tet-rbf1 constructs. For PCNA-luciferase assay, 1.5 million cells were transfected with 1 μg of PCNA-luciferase reporter, 250 ng of pRL-CMV *Renilla* luciferase reporter (Promega), and 200 ng of pAX Rbf1-WT, pAX Rbf1-ΔIE, or pAX-Ub-Rbf1-ΔIE constructs. 1000 ng of pAX-Ub-Rbf1-WT and 3 ng of pAX Rbf1-WT were used in Fig. 6*B*. Cells were harvested 3 days after transfection, and luciferase activity was measured using the Dual-Glo luciferase assay system (Promega) and quantified using the Veritas microplate luminometer (Turner Biosystems). Firefly luciferase activity was normalized to *Renilla* luciferase activity except when analyzing Rbf1 activity on the InR promoter. For doxycycline treatment (1 μg/ml), the drug was added to the media immediately after transfection.

Western Blot Analysis—To measure protein levels in S2 cell culture, cells were harvested 3 or 5 days after transfection and lysed by freeze-and-thaw cycles three times in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100). Typically, 50 μg of S2 cell lysates were separated by 12.5% SDS-PAGE, transferred to PVDF membrane for analysis using M2 anti-FLAG (mouse monoclonal, 1:10,000, Sigma, F3165), anti-GFP (mouse monoclonal, 1:1,000, Santa Cruz Biotechnology, sc-9996), and anti-tubulin (mouse monoclonal, 1:20,000, Iowa Hybridoma Bank). Antibody incubation was performed in TBST (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 0.1% Tween 20) with 5% nonfat dry milk. Blots were developed using HRP-conjugated secondary antibodies (Pierce) and SuperSignal West Pico chemiluminescent substrate (Pierce).

Stability Assays—For determination of GFP fusion protein half-life, 1.5 million S2 cells were transfected with 200 ng of pAX-GFP-Rbf1-IE or 400 ng of pAX-GFP. After a 3-day incubation, cells were treated with 100 μM cycloheximide for the indicated times. For proteasome inhibitor treatments in Figs. 2*B* and 6*A*, 72 h after transfection, cells were treated with DMSO or DMSO containing 50 μg/ml MG132 (Sigma-Aldrich) for 2 h.

In Vivo Ubiquitination Assay—In experiments shown in Fig. 2, *A* and *B*, S2 cells were co-transfected with 250 ng of pAX Rbf1-WT, 250 ng of pAcGal4, and 250 ng of UAS-Ub constructs using Effectene transfection reagent (Qiagen). In Fig. 3*A*, cells were transfected with 50 ng pAX Rbf1-WT or pAX Rbf1-ΔIE, 50 ng of pAcGal4, and 50 ng of UAS-Ub constructs. In Fig. 3*B*, cells were transfected with 200 ng of Rbf1 WT, 400 ng of pAX-GFP-FLAG, and 200 ng of pAX GFP-Rbf1-IE constructs. In all cases, cells were grown for 3 days, after which extracts were prepared using SDS lysis buffer (2% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0). The extracts were heat-denatured and sonicated followed by a 10-fold dilution using dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100). FLAG immunoprecipitation reactions were performed (anti-FLAG M2 affinity gel, Sigma) fol-

lowed by anti-HA Western blotting (mouse polyclonal antibody, 1:5000 dilution).

RESULTS

A Modular Degron Influences Rbf1 Ubiquitination and Stability—Drosophila Rbf proteins are subjected to developmentally regulated turnover, exhibiting tissue-specific modulation in both the developing embryo and the larvae (20, 28). To understand the mechanism underlying this regulation, we tested whether the Rbf1-IE can autonomously control protein stability by fusing the IE region (728–786) to GFP (Fig. 1*A*) and measuring the half-lives of GFP and GFP-Rbf1-IE chimeras in S2 cells after cycloheximide treatment. Steady state levels of GFP-Rbf1-IE, but not GFP, were substantially decreased by 12 h after cycloheximide challenge, indicating that the IE directly enhanced GFP turnover (Fig. 1*B*). Thus, the IE region can function autonomously as a degron and independently of other domains within Rbf1. This ability is consistent with the previously discovered role of the IE in control of full-length Rbf1 stability during development (20).

Previous models of degron function indicate that subcellular location of substrate proteins influences turnover (29). Therefore, to examine the effect of substrate localization on Rbf1 degron function, the Rbf1 nuclear localization signal (NLS, supplemental Fig. S1) was appended to GFP-Rbf1-IE, largely confining the chimera protein to the nucleus (Fig. 1*C*). Accumulation of the GFP chimera proteins was then measured, testing lysine-to-alanine substitutions within the IE that were previously shown to both inactivate and stabilize wild type Rbf1 (20). In all experiments, both GFP-Rbf1-IE (−NLS) and GFP-Rbf1-C (+NLS) behaved similarly, with Lys-to-Ala mutants accumulating to levels ~3-fold higher than those of their wild type counterparts. Consistent with these observations, the GFP-Rbf1-IE 4KA mutant displayed a significantly longer half-life as compared with GFP-Rbf-IE (supplemental Fig. S2). The steady state levels of both GFP-Rbf1-IE and GFP-Rbf1-C were unaffected by lysine-to-arginine substitution of the same amino acids, indicating that the positive charges of the side chains are important for IE substrate destabilization and that these lysine residues are unlikely targets for ubiquitination (Fig. 1*D*). These data indicate that the function of the IE as a modular degron is unaffected by its preferential nuclear localization and is consistent with a model wherein some components of the Rbf1 degradation pathway occur in the nucleus.

Regulated protein turnover often involves the activity of the 26S proteasome, which interacts with substrates that have been modified with ubiquitin, but also in some cases proteins that are not ubiquitinated. In mammals, RB and p107 are substrates of E3 ubiquitin ligases and are turned over in a proteasome-dependent manner (30–33). Rbf1 is likewise dependent on the proteasome pathway, but there are no reports of ubiquitination of this protein. To test whether Rbf1 is ubiquitinated *in vivo*, we expressed FLAG-tagged Rbf1 and HA-tagged ubiquitin proteins in S2 cells and immunoprecipitated the Rbf1 proteins. As shown in Fig. 2*A*, polyubiquitinated Rbf1 species were detected in heat-denatured extracts prepared from cells co-expressing both FLAG-Rbf1 and HA-ubiquitin. Ubiquitinated species were not observed in mock-transfected samples, in samples

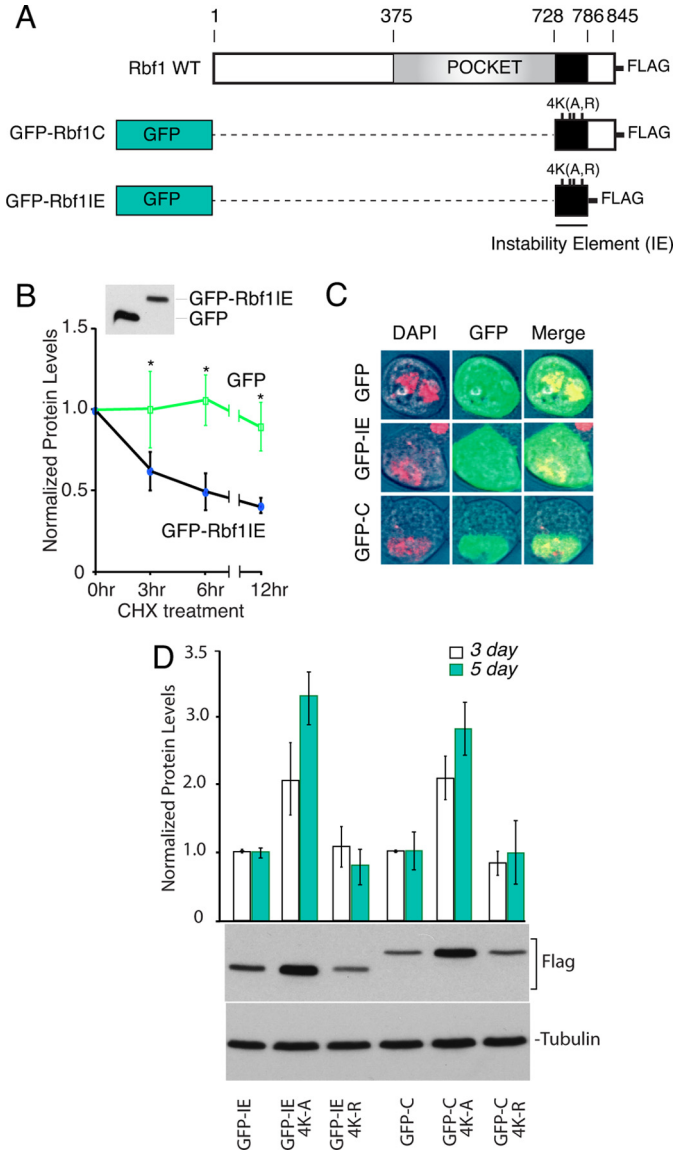

FIGURE 1. **The IE of Rbf1 is a modular degron.** *A*, schematic diagram of GFP fusion proteins expressed in *Drosophila* S2 cells. *B*, the presence of the IE increases protein turnover. Half-lives of GFP fusion proteins were measured by Western blot after cycloheximide (*CHX*) treatment (*error bars* indicate S.D., $p < 0.01$). *Inset*, Western blot shows the steady state levels of GFP and GFP IE fusion protein before cycloheximide treatment. *C*, subcellular localization of GFP and GFP fusion proteins as measured by confocal microscopy. *D*, IE function modulates GFP stability. Indicated GFP fusion proteins were expressed in S2 cells for 3 or 5 days and measured by Western blot with antibodies against the FLAG epitope. Lysine residues (Lys-732, Lys-739, Lys-740, and Lys-754) were changed to alanine or to arginine. Protein levels were quantitated by photon capture analysis with a Fuji LAS-3000 imager and normalized to tubulin levels. *Error bars* indicate S.D., and *asterisks* indicate $p < 0.01$. Western blot data are representative from the 5-day set of experiments.

containing only one of the two proteins, or in extracts containing Rbf1 and HA-ubiquitin from denatured extracts containing individually expressed HA-Ub or FLAG-Rbf1 proteins that were mixed together prior to immunoprecipitation. In the presence of the MG132 proteasome inhibitor, higher levels of polyubiquitinated Rbf1 were observed (Fig. 2*B*). We conclude that the Rbf1 protein was ubiquitinated *in vivo* and is targeted for proteasome-mediated turnover, an outcome that is consistent with previous observations linking the COP9 signalosome to protection of Rbf1 from destruction by the proteasome (24).

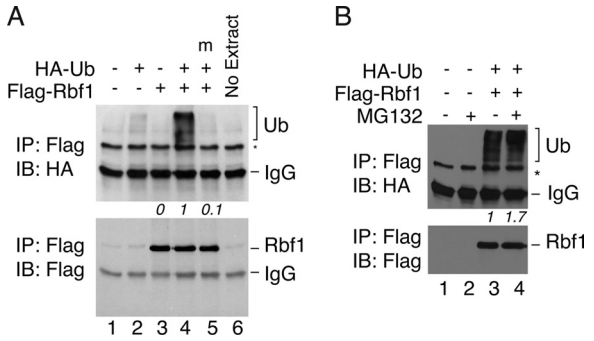

FIGURE 2. **Rbf1 is degraded via a ubiquitin-proteasome-dependent pathway.** *A*, Rbf1 is ubiquitinated *in vivo*. S2 cells were transfected with FLAG-tagged Rbf1 and HA-tagged ubiquitin expression constructs. Denatured protein extracts were used for FLAG immunoprecipitation (*IP*), and recovered samples were assayed by anti-HA Western blot (*IB*) analysis (*top panel*). The *asterisk* indicates a nonspecific band, and *m* indicates a reaction performed using mixed samples from those in *lanes 2* and *3*. The immunoprecipitation samples were also blotted with anti-FLAG antibody (*bottom panel*) to verify equivalent Rbf1 recovery (*lanes 3–5*). The *numbers* underneath the HA Western blot panel represent the ratios of HA/FLAG signals. The data shown are representative of three biological replicates. *B*, Rbf1 ubiquitination is sensitive to proteasome inhibition. Samples were treated as in *A* except that they were treated with MG132, a proteasome inhibitor.

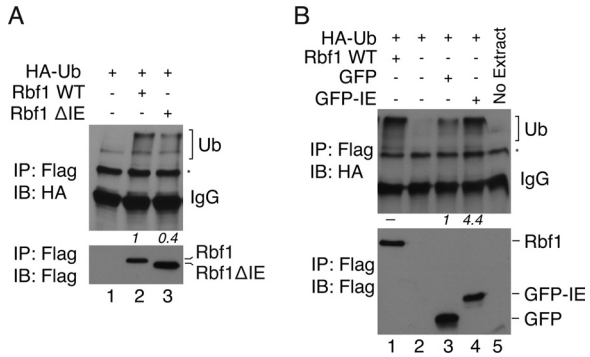

FIGURE 3. **The Rbf1 instability element enhances protein ubiquitination.** *A*, the Rbf1 IE enhances ubiquitination. Wild type and mutant Rbf1 lacking the IE (Rbf1-ΔIE) were compared for ubiquitination as performed in Fig. 2. *IP*, immunoprecipitation; *IB*, Western blot. The *asterisk* indicates a nonspecific band. *B*, the Rbf1 IE is sufficient to drive the ubiquitination of a heterologous protein, GFP. Fusion of the Rbf1-IE to GFP led to a substantial increase in the levels of its ubiquitination as compared with the levels observed for GFP as measured by co-transfection and co-immunoprecipitation/Western analysis.

Interestingly, Rbf1 lacking the IE region (Rbf1-ΔIE) exhibited a substantial reduction, but not complete loss, of Rbf1 ubiquitination (Fig. 3*A*), a result that was also observed for Rbf1-4KA (supplemental Fig. S3), suggesting that the IE enhances ubiquitination, but is not essential for all modification events. We tested whether the IE is sufficient to independently drive ubiquitination by co-expressing HA-tagged ubiquitin and the GFP-IE chimera. Indeed, as shown in Fig. 3*B*, levels of polyubiquitinated GFP were substantially increased by appending the Rbf1-IE region as compared with levels observed for untagged GFP. GFP-Rbf1 IE ubiquitination was reduced by the introduction of the 4KA substitutions (supplemental Fig. S4). Together, these data show that one function of the Rbf1 IE is to facilitate substrate ubiquitination.

The Rbf1-IE Can Function Independently in Transcriptional Repression—We showed previously that in addition to influencing protein stability, the IE region is critical for Rbf1 repressor activity on E2F1-dependent promoters, such as *PCNA* and *Polα* (20). We therefore hypothesized that the Rbf1 degron functions as a *bona fide* transcriptional repression domain. To test this hypothesis, the Rbf1 degron alone or degron plus NLS was fused to the Tet repressor, and the activity of these proteins was assayed on an Actin5C reporter harboring two Tet binding sites (Fig. 4*A*). Indeed, when directly tethered to its target promoter in the absence of doxycycline, both Tet-Rbf1-IE and Tet-Rbf1-C showed strong repression activity at levels approaching that observed with Tet-Knirps, a potent short range repressor that was included as a positive control on this reporter (Fig. 4*B*). As expected, treatment with doxycycline to inhibit DNA binding also diminished repression (not show). The Tet repressor DNA binding domain alone lacked notable repression activity. These data are consistent with a direct role for the IE in transcriptional repression. Interestingly, both Tet-Rbf1-C and Tet-Rbf1-IE harboring the Lys-to-Ala substitutions repressed transcription to similar levels as observed for the wild type Tet-Rbf1-IE chimera. Thus, these lysine residues that influence repression in the context of full-length Rbf1 are not essential in this context (20).

The ability of the IE to independently repress transcription next prompted us to examine whether the IE is an essential element within full-length Rbf1 when targeted to a promoter independently of E2F1. Strikingly, the Tet-Rbf1 chimera lacking the IE (Tet-Rbf1-ΔIE) was not compromised for activity; the protein repressed transcription from the Actin5C-Tet reporter as effectively as did the wild type Tet-Rbf1 chimera, indicating that the IE is not essential in this context (Fig. 4*C*). When assayed on the PCNA reporter that lacks Tet binding sites but utilizes E2F1 to recruit Rbf1, the Tet-Rbf1-ΔIE chimera was compromised for repression, consistent with previous observations that the IE is important for Rbf1 repression of cell cycle genes (20). Therefore, this outcome suggests that the mechanism of promoter targeting does influence whether the IE region functions in repression. Interestingly, both Tet-Rbf1-C (4KA) and Tet-Rbf1-IE (4KA) were expressed at similar levels as their wild type counterparts and under conditions wherein the same alanine substitutions increased Tet-full-length Rbf1 steady state levels (Fig. 4*D*). These observations suggest that the function of these IE-lysine residues is context-dependent for both repression and stability.

Context-dependent Repression by Rbf1-IE Regulatory Domain—The substantial repression exhibited by the Rbf1-ΔIE mutant protein when directly recruited to the Tet promoter demonstrated that this protein is not inherently defective. This observation also raised the interesting possibility that the IE provides gene-specific repression capability. To examine the possibility that the IE provides repression capability specifically in the context of E2F1-regulated promoters, the repression potency of wild type Rbf1 was compared with Rbf1-ΔIE on E2F1-regulated promoters (*PCNA*, *Polα*, and *Mcm7*) (Fig. 5*A*) and noncanonical E2F1-independent promoters (*InR*, *wts*, and *Pi3K68D*) (Fig. 5*B*). The *InR*, *wts*, and *Pi3K68D* gene promoters are devoid of recognizable E2F1 binding sites and were refractory to activation by E2F1, but are directly bound by Rbf1 in the embryo (22). On the canonical target genes, Rbf1-ΔIE was much weaker than wild type Rbf1 for E2F1-dependent gene repression, but both

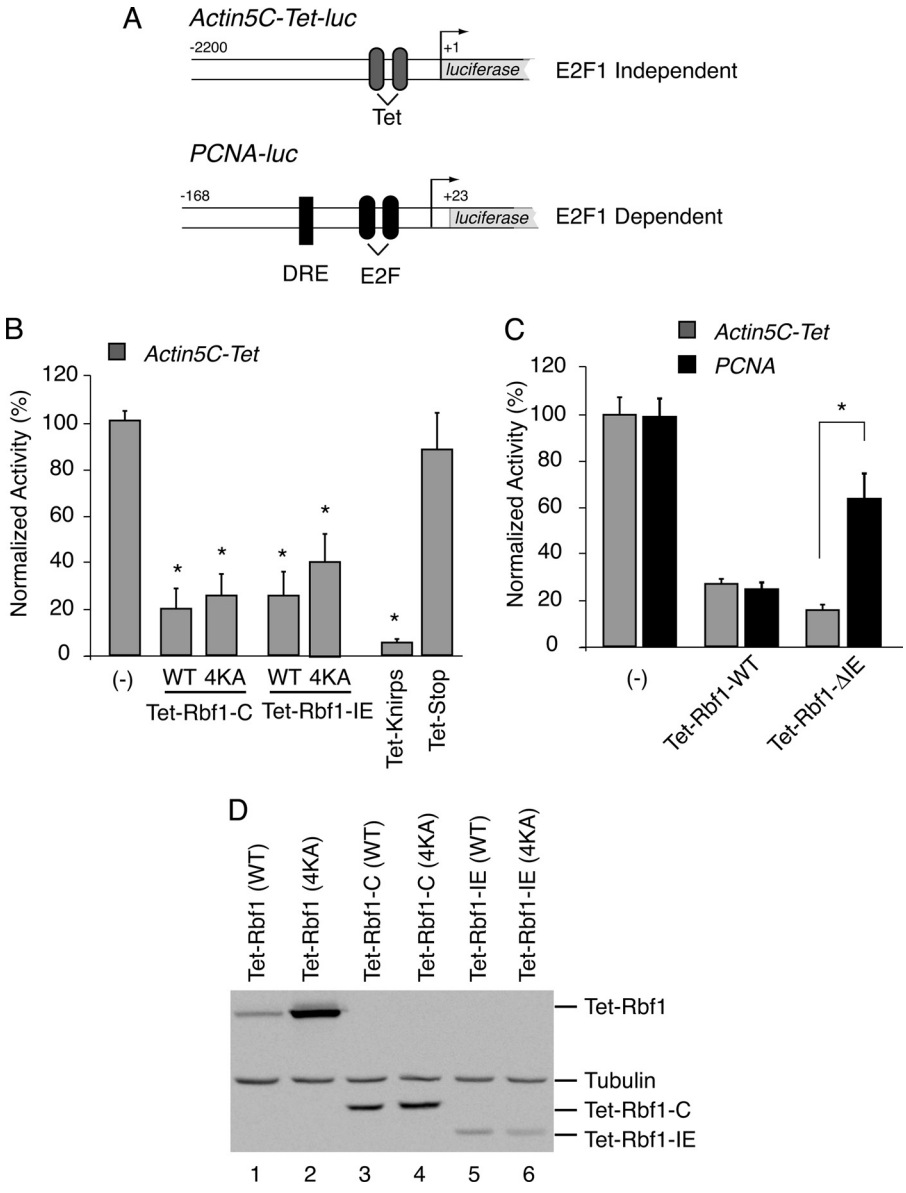

FIGURE 4. **Rbf1 IE functions as a transcriptional repression domain.** *A*, schematic representation of the E2F1-independent and E2F1-dependent reporter genes used in this study. *B*, transcriptional activities of Tet fusion proteins were assayed on the *Actin5C*-Tet-luc reporter. The IE with or without the NLS repressed the target gene when directly tethered to the promoter as compared with reactions lacking Rbf1 fusion proteins (*, $p < 0.05$). Both the WT and the 4KA mutant versions repressed transcription equivalently. A Knirps fusion protein (Tet-Knirps) and Tet protein alone (Tet-Stop) served as positive and negative controls, respectively. *Error bars* indicate S.D. *C*, transcriptional activities of the Tet-Rbf1 WT and Tet-Rbf1 ΔIE chimeras were compared on the *Actin5C*-Tet-luc and *PCNA*-luc reporters. Data are from at least three biological replicates. *Error bars* indicate S.D. *D*, levels of the indicated Tet-Rbf1 fusion proteins were determined by anti-FLAG Western blot analysis 3 days after transfection. Lysine-to-alanine substitution did not affect steady state levels of the Tet-Rbf1-IE and Tet-Rbf1-C proteins under conditions wherein Tet-Rbf1 levels were increased. Tubulin levels are shown as a loading control.

repressors exhibited similar potency on the noncanonical Rbf1 reporter genes. As previous data showed that Rbf1-ΔIE can interact with E2F1 and associate with endogenous E2F1 target genes (22), the IE may provide post-recruitment functions that are dispensable when Rbf1 is recruited independently of E2F1.

Rbf1 Ubiquitination Stimulates Repressor Potency—The function of the instability element as both a repression domain and a degron that stimulates Rbf1 ubiquitination suggested that ubiquitin might function directly in Rbf1-mediated repression. We showed above that MG132 treatment substantially increases the levels of ubiquitinated Rbf1. Therefore, we measured Rbf1-mediated repression of the PCNA reporter in the presence or absence of MG132 (Fig. 6*A*). A modest but reproducible enhancement in repression potency of wild type Rbf1 was observed within 2 h of drug treatment, an effect that was not observed with the Rbf1-ΔIE mutant. These data are consistent with IE-directed ubiquitination influencing repression activity. Although MG132 affected only the wild type Rbf1, a general concern remained that global proteasome inhibition may induce pleiotropic effects (34). Therefore, to directly assess the effect of ubiquitin on Rbf1 function, repression assays were performed using chimera proteins containing ubiquitin fused to the N terminus of full-length Rbf1. As ubiquitin attachment markedly destabilized full-length Rbf1 (see also Fig. 6*C*), consistent with this modification directing Rbf1 for proteasome destruction, repression assays were performed using differing

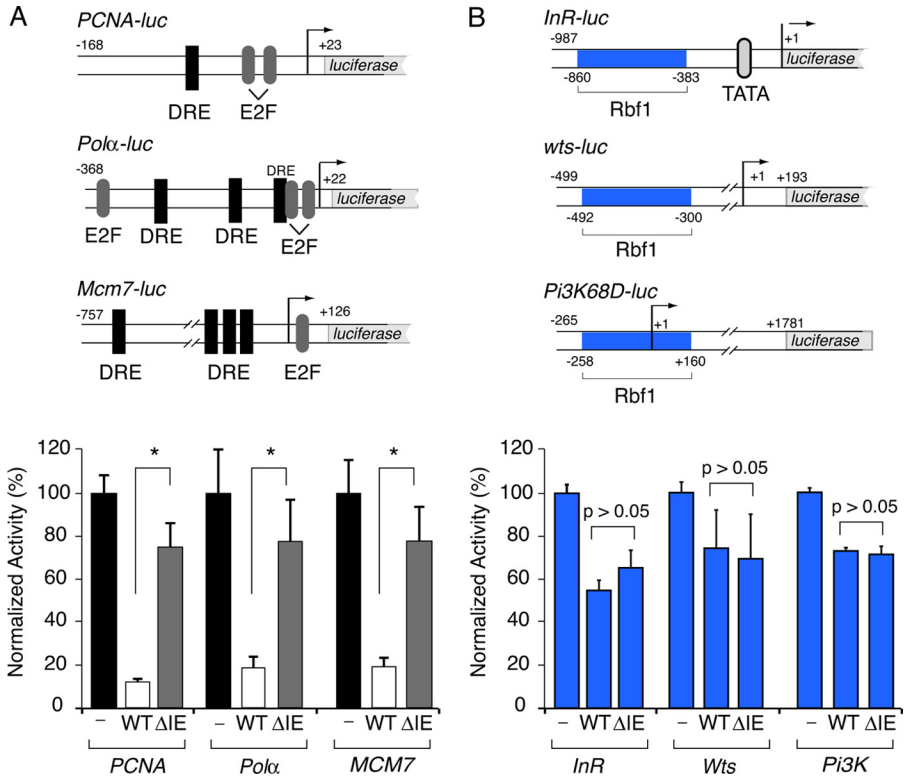

FIGURE 5. **Context dependence of the Rbf1-IE for transcriptional repression.** *A* and *B*, Rbf1 WT and Rbf1 ΔIE showed dissimilar repression activities on the E2F1-dependent reporters as compared with the E2F1-independent promoters. Transcriptional activity was measured as described in the legend for Fig. 4. Data are from at least three biological replicates. *, $p < 0.05$, *error bars* indicate S.D.

amounts of expression plasmids to equalize repressor concentration. Under conditions wherein both Rbf1 and Ub-Rbf1 were expressed at comparable levels, the presence of ubiquitin markedly improved Rbf1 repression activity on the PCNA promoter on average 4–5-fold (Fig. 6*B*). This outcome supports the hypothesis that ubiquitin can contribute directly to target gene repression.

The potent role of ubiquitin in Rbf1 target gene repression noted above allowed the possibility to examine whether polyubiquitination at this site is essential for enhanced repressor potency. To test this possibility, K48R and K63R substitutions were incorporated within the N-terminal ubiquitin at positions expected to impede polyubiquitination. Indeed, as shown in Fig. 6*C*, Rbf1 appended with mutant ubiquitin (K48R and K63R) was maintained at higher steady state levels than Rbf1 fused to wild type ubiquitin when expressed using comparable amounts of expression plasmid. Thus, the N-terminal ubiquitin was functional in the proteasome-mediated turnover of Rbf1. When compared with wild type Rbf1 lacking ubiquitin, Rbf1 harboring mutant ubiquitin remained a more potent repressor of PCNA transcription. This result suggests that although ubiquitination at the Rbf1 N terminus can contribute to repression potency, polyubiquitination at this site is not essential for this enhancement. Nonetheless, in all experiments, Rbf1 containing wild type ubiquitin did exhibit improved specific activity, suggesting that higher order ubiquitination can contribute to repression.

Based on the observation that Rbf1-ΔIE is defective for repression on E2F1 target genes, whether the forced ubiquitination of Rbf1-ΔIE could stimulate repression potency was tested. In this experiment, higher levels of Rbf1-ΔIE were tested to ensure that active proteins were being compared. Under these conditions, and despite substantially lower steady state protein levels associated with forced ubiquitination, Rbf1-ΔIE harboring the appended wild type ubiquitin exhibited increased repression ability of PCNA transcription (Fig. 6*D*). However, ubiquitin did not enhance Rbf1-ΔIE repression of the InR reporter, suggesting that the effect of this modification is restricted to certain types of target genes. These observations imply that insufficient ubiquitination observed with IE deletion underlies the loss of repression activity at cell cycle-regulated genes.

DISCUSSION

The RB family of proteins governs diverse physiological processes including cell cycle, apoptosis, and differentiation. An important question remains how these factors maintain differential influence over mutually exclusive pathways. Previous studies demonstrated that mammalian RB phosphorylation by cell cycle-dependent kinases or stress-responsive kinases can distinguish between cell cycle arrest or apoptotic responses (16). In this study of the *Drosophila* Rbf1 protein, we uncovered a direct role for ubiquitination in differential gene regulation. In particular, the C-terminal regulatory domain of Rbf1 was found to harbor an independently acting degron that directs Rbf1 ubiquitination. Post-translational modification by ubiquitin improved Rbf1 transcriptional repression, directly linking repressor potency to ubiquitin-mediated turnover pathways. Furthermore, Rbf1 lacking the degron was also debilitated for repression of cell cycle-regulated *PCNA*, *Polα*, and *Mcm7* pro-

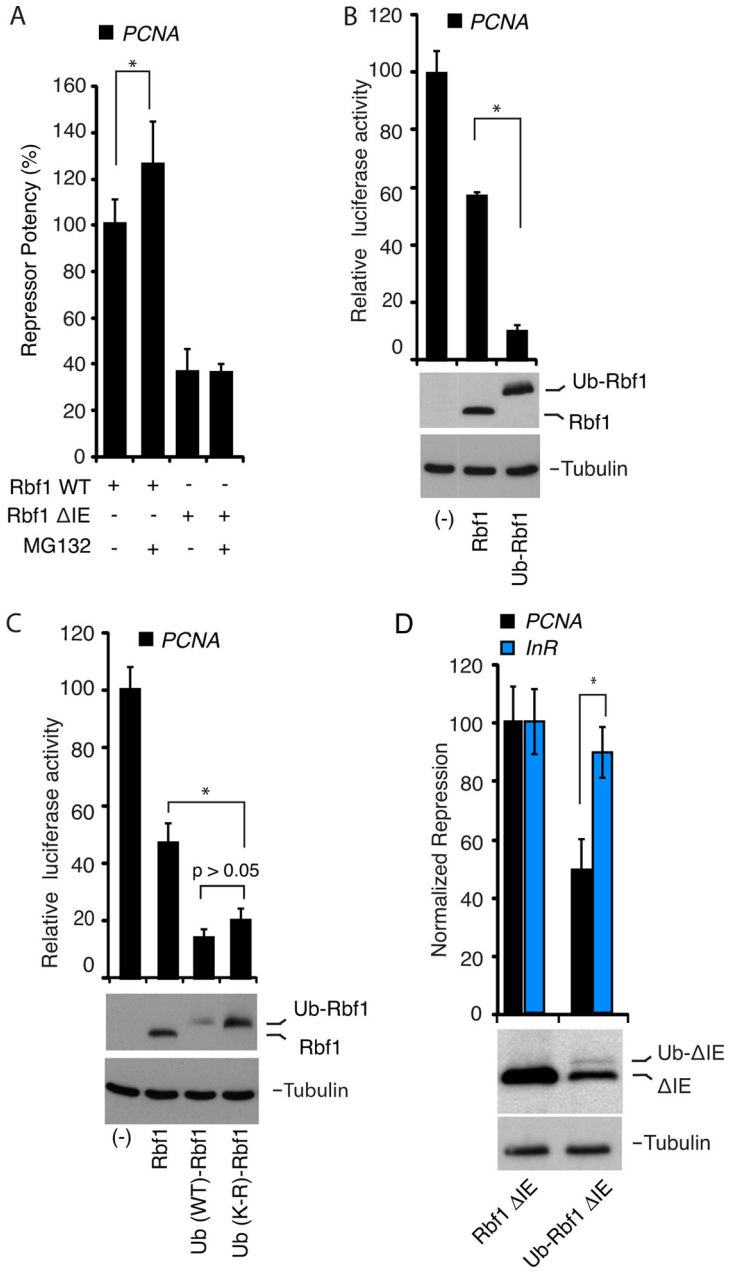

FIGURE 6. **Rbf1 ubiquitination enhances gene-specific repression activity.** *A*, proteasome inhibition by MG132 influences transcriptional repression activity of Rbf1 on the PCNA-luc reporter. Repression potency of WT Rbf1 on the *PCNA*-luc reporter (set to 100%), but not the ΔIE mutant, was significantly enhanced after MG132 treatment (*, $p < 0.01$). *B*, ubiquitin enhances Rbf1 repression potency. In this experiment, wild type Rbf1 expression was adjusted to match that of the unstable Ub-Rbf1 chimera (3 ng of pAX-Rbf1 WT *versus* 1000 ng of pAX-Ub-Rbf1 WT) for testing using the *PCNA*-luc reporter (*upper panel*). At comparable levels of repressor, as detected by FLAG Western analysis (*lower panel*), ubiquitin improved Rbf1-specific activity 3– 4-fold. Tubulin levels are shown as a loading control. *C*, polyubiquitination of the N-terminal ubiquitin is not essential for enhanced repression. Lys-to-Arg substitutions at positions 48 and 63 within the N-terminal ubiquitin tag increased Rbf1 steady state levels as compared with wild type ubiquitin-Rbf1 chimeras in transfection experiments using equal amounts of DNA (*lower panel*). At comparable protein levels, the mutant Ub-Rbf1 chimera repressed transcription better than Rbf1 lacking the ubiquitin tag (*, $n = 3$, $p < 0.05$) and to levels similar to those observed for the Rbf1 chimera harboring the wild type ubiquitin tag. *D*, ubiquitin fusion partially restores transcriptional repression activity to Rbf1-ΔIE on the *PCNA*-luc reporter ($p < 0.05$) but not on the *InR*-luc reporter using equal amounts of DNA during transfection. In these experiments, the faster migrating protein observed with the Ub-Rbf1-ΔIE fusion protein (*lower panel*) is likely due to substantial cleavage of the ubiquitin tag. *Error bars* indicate S.D.

moters, but not for regulation of noncanonical Rbf1 target genes, thus highlighting a role for ubiquitination in differential regulation of Rbf target genes. These findings point to distinct modes of transcriptional repression depending upon the promoters targeted. Recent genomic studies have shown that Rbf1 association at many noncanonical promoters, including the *InR* locus, is independent of E2F1 but is dependent upon the general E2F partner, DP1 (23). Thus, it remains possible that the Rbf1 degron functions primarily when recruited by E2F1-DP1 and not when recruited by E2F2-DP1. This concept is consistent with structural studies of human RB that show the corresponding region located within the RB C terminus is important for interactions with E2F1/DP1 complexes (35). As the Rbf1 degron sequence is highly conserved within the mammalian RB

homologs p107 and p130, degron function in differential gene repression may be evolutionarily conserved.

Although ubiquitin clearly enhanced Rbf1 activity toward the *PCNA* promoter, the molecular mechanism by which ubiquitination is associated with transcriptional repression is unknown. In one model, repression is enhanced by direct proteasome recruitment to a promoter through interactions mediated by ubiquitin. In a second model, ubiquitination serves two roles, recruiting essential cofactors to a promoter and separately interacting with the protein degradation machinery. Aspects of this mechanism are analogous to the degron theory of gene activation previously described for the c-Myc proto-oncoprotein (36–39). During activation, ubiquitin can function for co-factor recruitment, such as described for recruitment of P-TEFb by the viral activator VP16 (40), and thus ubiquitin may similarly contribute to RB co-repressor recruitment. As our studies demonstrate that the C-terminal degron may recruit an E3 ligase, a direct role for these enzymes in Rbf1 gene regulation is possible. Such a direct role for E3 ligases in repression was observed for BRCA1-mediated transcriptional regulation (41); however, in that example, ubiquitin interfered with assembly of the preinitiation complex. Alternatively, Rbf1-mediated E3 recruitment could promote E2F1 ubiquitination. However, the IE region does not appear to influence Rbf1-mediated E2F1 stabilization (42). Whether E3 ligases participate directly in Rbf1-mediated repression is unknown; nonetheless, observations that the COP9 signalosome, an evolutionarily conserved complex that functions to inhibit E3 ligase activity, was directly found at Rbf1 target genes simultaneously with the Rbf1 repressor (24) suggest that a complex network of feedback regulation is proximally available at Rbf1 target gene promoters.

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